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The PAX5 oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a S-type cell line

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Abstract: Neuroblastoma is a neural crest-derived neoplasm of infancy with poor outcome in patients with advanced disease. The oncogenic transcription factor PAX5 is an important developmental regulator and is implicated in the pathogenesis of several malignancies. Screening of neuroblastoma cell lines revealed PAX5 expression in a malignant subset of neuroblastoma cells, so-called 'N-type' cells, but not in the more benign 'S-type' neuroblastoma cells. PAX5 expression was also detected in small cell lung cancer, an aggressive tumor of neural crest origin. Based on this observation we hypothesized that there could be a relationship between PAX5 expression and the more malignant phenotype of N-type cells. Stable PAX5 expression was established in several clones of the S-type cell line CA-2E. A noticeable difference in morphology of these transfectants was observed and there was also a significant increase in the proliferation rate. Moreover, PAX5 expressing clones gained the ability to form colonies in a soft agar assay, a marker of tumorigenicity. Down-regulation of PAX5 in several N-type cell lines and one small cell lung cancer cell line utilizing small interfering RNA resulted in a significant decrease in growth rate. Taken together we propose PAX5 as an important factor for the maintenance of the proliferative and tumorigenic phenotype of neuroblastoma. Our data, together with a recent study on the role of PAX genes in cancer suggest that PAX5 and other PAX transcription factors might be valuable targets for cancer therapy

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The *PAX5* oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a S-type cell line

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Neuroblastoma is a neural crest-derived neoplasm of infancy with poor outcome in patients with advanced disease. The oncogenic transcription factor *PAX5* is an important developmental regulator and is implicated in the pathogenesis of several malignancies. Screening of neuroblastoma cell lines revealed *PAX5* expression in a malignant subset of neuroblastoma cells, so-called 'N-type' cells, but not in the more benign 'S-type' neuroblastoma cells. *PAX5* expression was also detected in small cell lung cancer, an aggressive tumor of neural crest origin. Based on this observation we hypothesized that there could be a relationship between *PAX5* expression and the more malignant phenotype of N-type cells. Stable *PAX5* expression was established in several clones of the S-type cell line CA-2E. A noticeable difference in morphology of these transfectants was observed and there was also a significant increase in the proliferation rate. Moreover, *PAX5* expressing clones gained the ability to form colonies in a soft agar assay, a marker of tumorigenicity. Down-regulation of *PAX5* in several N-type cell lines and one small cell lung cancer cell line utilizing small interfering RNA resulted in a significant decrease in growth rate. Taken together we propose *PAX5* as an important factor for the maintenance of the proliferative and tumorigenic phenotype of neuroblastoma. Our data, together with a recent study on the role of *PAX* genes in cancer suggest that *PAX5* and other *PAX* transcription factors might be valuable targets for cancer therapy.

Introduction

Neuroblastoma is the most common extracranial solid tumor in children and is the leading cause of cancer death in children between 1 and 4 years (1,2). It is a neoplasm of the neural crest, a transient embryonic structure giving rise to a variety of tissues (e.g. melanocytes, neurons, neuroendocrine tissue and

smooth muscle cells) (3). Histologically neuroblastoma tumors are composed of distinct malignant cell types, including neuroblastic and stromal cells (1).

Cellular diversity is also characteristic of cell lines established from explanted neuroblastoma tumors. A number of subpopulations have been identified in these cultures, including neuroblastic (N-type), substrate adherent (S-type) cells and cells with an intermediate phenotype (I-type). These cell types can be distinguished by a characteristic morphology (4–6). Analysis of homogeneous cultures of these subtypes has shown that they can also be distinguished by the expression of a number of differentiation-associated markers. N-type cells express neurotransmitter biosynthetic enzymes, chromogranin A and neurofilament proteins while S-type cells synthesize collagen, fibronectin and vimentin (4,7). In contrast to S-type cells, N-type cells are tumorigenic in nude mice and form colonies in soft agar (8). The difference in malignant properties between S- and N-type cell subtypes may have clinical relevance (9). At the transcriptional level several differences in gene expression have been detected. For example, *BCL2* and *MYCN* are usually expressed at a higher level in N-type cells (10,11). The difference in *MYCN* expression was observed in both *MYCN* amplified and non-amplified cell lines (11,12). Recently it has been demonstrated that S-type in contrast to N-type cells are more susceptible to TRAIL-mediated apoptosis due to their expression of caspase-8 (13,14).

PAX5 is a member of the *PAX* gene family of developmentally regulated transcription factors that play a fundamental role in organogenesis (15). All *PAX* transcription factors share a DNA binding domain—the paired box. Their importance in development has been underscored by several loss-of-function mutations that cause lack of a specific structure or organ in which the corresponding *PAX* gene is normally expressed. Typically, *PAX* protein expression is down-regulated in the adult organism. During mouse brain development *Pax5* is expressed predominantly at the midbrain–hindbrain boundary. A weaker expression is found along the entire neural tube, particularly in the ventricular zone of both alar and basal plates (16). A similar expression pattern has been described recently in human embryos (17). In addition, *Pax5* is expressed during all stages of B-cell development, except in plasma cells (18). *Pax5*^{−/−} mice lack mature B cells and show defects of the inferior colliculus and anterior cerebellum (19).

In addition to the normal physiological function during development, *PAX* transcription factors have been shown to play an important role in tumorigenesis (reviewed in ref. 20). First, a number of *PAX* genes are located at recurring, tumor-specific chromosomal translocations, suggesting that *PAX* genes have an oncogenic capacity when inappropriately expressed. Secondly, several *PAX* genes are re-expressed in malignant neoplasms, usually in tumors derived from tissue in which the respective *PAX* gene is expressed during development. For example, deregulated expression of *PAX5* has been described in medulloblastoma and lymphomas (21,22) and of

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; scRNA, scrambled control; siRNA, small interfering RNA.

PAX3 in melanoma (23). It is speculated that the re-expressed *PAX* genes promote tumor development and progression by increasing proliferation and motility, while inhibiting apoptosis (20).

Here we describe that *PAX5* is expressed in aggressive N-type neuroblastoma cell lines, while no expression was detected in S-type cells. Over-expression of *PAX5* in the S-type cell line CA-2E restored several malignant properties, in particular anchorage-independent growth. In addition down-regulation of *PAX5* in several N-type cell lines significantly reduced their proliferation rate. These results provide additional evidence for an important role of the *PAX* gene family in tumor development and suggest a role for *PAX5* in neuroblastoma disease progression.

Materials and methods

Cell lines

ACN, CA-2E, BE(2)C, BE(2)M17, Imr32, Kelly, LAN-1, LAN-5, SH-310, SH-EP, SH-IN, SK-N-BE(2), SH-SY5Y and WSN were kindly provided by Nicole Gross (University Hospital, Lausanne, Switzerland). U1285 was a kind gift of Larisa Belyanskaya (Universitaetsspital, Zurich, Switzerland), MML1157 was provided by Juerg Zarn (formerly Universitaetsspital Zurich, Switzerland) and Kis-1 was kindly provided by Hitoshi Ohno (Kyoto University, Kyoto, Japan). SW2, OH1 and OH3 have been described previously (24). A549, Calu1, Calu3, Calu6, H460, NCI-H69 and NCI-H125 were obtained from ATCC (Rockville, MD). All cell lines were cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO₂.

RT-PCR

RNA was isolated from cell lines using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Basel, Switzerland). Five micrograms of total RNA were used to synthesize cDNA using the M-MuLV reverse transcriptase according to the manufacturer's instructions (MBI Fermentas, Vilnius, Lithuania). One-hundredth of the product was used for amplification using primers for *PAX5* or *GAPDH* and PCR conditions as described elsewhere (25).

Plasmid construction

A mammalian *PAX5* cDNA expression plasmid designated pX-13 was generated by cloning a 3.3 kb *XbaI* fragment from pBSAP-1s (a kind gift of Meinrad Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) into the mammalian expression vector pcDNA3.1/Hygro(+) (Invitrogen, Basel, Switzerland) using standard methods (26). This fragment contained the full-length *PAX5* coding sequence (1176 bp) together with 77 bp of the 5'-UTR and 2026 bp of the 3'-UTR sequence. The correct orientation of the *PAX5*-cDNA was confirmed by sequencing (Microsynth, Balgach, Switzerland).

Cell transfection and generation of stable cell lines

Transfection of the plasmids was performed with Superfect following the manufacturer's instructions (Qiagen, Basel, Switzerland). Stable clones were generated by transfecting CA-2E cells with plasmids pX-13 or pcDNA3.1/Hygro(+) for 20 h. The transfection medium was aspirated and cells were cultured in fresh medium for another day before adding 400 µg/ml hygromycin B for selection. Single clones were achieved by plating the cells in 96-well plates after 7–10 days of selection at a density of 0.5 cells/well in 100 µl selective medium. Single clones were expanded and screened for *PAX5* expression by immunoblotting.

Cell lysis and immunoblotting

Cells were harvested and pellets were frozen at –80°C for at least 1 h. RIPA buffer was used for cell lysis as described (22). Between 80 and 120 µg of total lysate was separated by SDS-PAGE on a 10% gel. Transfer and immunoblotting conditions have been described previously (24). For *PAX5* and actin detection the Pax5-C14 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-Actin antibody (ICN Biomedicals GmbH, Eschwege, Germany) have been used respectively and were combined with the corresponding secondary antibodies. Chemoluminescent signals were detected using the ECL Kit (Amersham Biosciences, Buckinghamshire, UK). Band intensities were measured using standard methods.

Growth experiments

5000 cells were plated in 6-well plates containing 3 ml RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were grown at standard conditions and harvested after 4 days. After centrifugation the cells were re-suspended in PBS and the number of cells was determined using a Coulter counter (Beckman Coulter, Fullerton, CA). The harvesting and counting procedure was repeated every 24 h for an additional 5 days. Experiments were performed in triplicate and repeated four times with similar results.

Soft agar assay

To determine the ability of anchorage-independent growth a soft agar clonogenic assay was performed with *PAX5* positive and negative clones. A base solution consisting of 1.5 ml 0.5% agar in culture medium was poured in each well of a 6-well plate. 3000 cells were re-suspended in 1.5 ml top solution consisting of 0.35% agar in culture medium and subsequently seeded on top of the polymerized base solution. All solutions were kept at 40°C before pouring to prevent early agar polymerization and to ensure survival of the cells. Plates were incubated at 37°C under standard culture conditions for 28–32 days and colony formation was visualized by staining with 0.01% crystal violet and quantified by counting. All experiments were performed in triplicate and experiments were repeated five times with similar results.

Xenografts

Six to eight week old female CD-1 (ICR nu/nu) mice (Charles River Laboratories, Sulzberg, Germany) were used and kept under specific pathogen-free conditions according to the guidelines of the Veterinary Office of the Kanton Zurich, Switzerland. Ten million cells re-suspended in sterile PBS were injected subcutaneously on the flanks. Tumor growth was assessed by measuring tumor size once per week.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor samples were subjected to immunohistochemical staining for *PAX5* and Vimentin as described elsewhere (27). The primary anti-Pax-5 (Transduction Laboratories, Lexington, KY) and anti-Vimentin (DAKO, Glostrup, Denmark) were used.

Transfection with small interfering RNA (siRNA) and measurement of cell proliferation

Briefly, for each experiment 5×10^5 cells/well were plated in 6-well plates and transfected with 80 nM siRNA or scrambled control RNA (scRNA) for 24 h using Lipofectamine 2000 as a transfection mediator according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). The sequences of the sense strand and of the complementary strand of the siRNA and scRNA were as follows: siRNA sense strand 5'-CGGCCACUCGCUUCCGGGCTT-3', complementary strand 5'-GCCCCGAAGCGAGUGGCCGTT-3' and scRNA sense strand 5'-GCUCGUGACUGCGCGCC-3', complementary strand 5'-GGCGCGCAGUCGACGGAGCTT-3'. These compounds were ordered as double-stranded RNA with a 2-nt DNA overhang (i.e. TT) at both 3'-ends (Prologo, Paris, France).

Cell proliferation was measured by use of a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (28). Twenty-four hours after treatment with siRNA or scRNA, cells were plated in 96-well plates and incubated in standard culture medium containing only 5% FCS under standard conditions. The amount of plated cells per well was as follows: LAN-1 and OH1 (1.5×10^4), Imr32 and MML1157 (2×10^4). The first MTT assay was performed immediately after distributing the cells in order to confirm the accuracy of the plating. Cell proliferation was assessed 24, 48, 72 and 96 h after plating. Data recording and analysis was performed as described previously (28). An individual standard calibration curve was constructed for each cell line and absorption values were equalized with the cell number by use of these standard curves.

Results

Expression of *PAX5* in N-type neuroblastoma and small cell lung cancer cell lines

Based on the observation that deregulated expression of *PAX* genes is often observed in tumors derived from tissues in which the respective *PAX* gene is expressed during normal organogenesis, we screened tumors of the nervous system for *PAX5* expression by RT-PCR. Expression of *PAX5* mRNA was detected in seven N-type and parental neuroblastoma cell lines while no transcripts were present in all four S-type

neuroblastoma cell lines and in two out of three I-type cell lines (Figure 1A). In order to compare the relative abundance of the two *PAX5* isoforms, which differ only in the first exon (29), we performed a quantitative Real-Time PCR with primers specific for isoforms 1A and 1B. All neuroblastoma cell lines that were positive for *PAX5* in the conventional RT-PCR expressed isoform 1B. It was only in a few cases that a very low expression of the 1A isoform could be detected as well (unpublished data). These findings are consistent with data showing that the 1B promoter is predominantly used for *PAX5* expression in non-B lymphocytes (30). *PAX5* expression in N-type and absence in S-type cells could be confirmed at the protein level by immunoblotting. A major band of the expected size of ~50 kDa was detected in lysates prepared from N-type

cell lines and the B-cell line Kis-1, but not in S-type cells. The minor bands above the band representing *PAX5* are non-specific and also visible in S-type cells. An actin control is included to ensure equal loading (Figure 1B).

As neuroblastoma is a neural crest-derived tumor, we also screened additional tumors of the neural crest for *PAX5* mRNA expression. While no expression could be detected in five melanoma cell lines (unpublished data), expression of *PAX5* was found in five small cell lung cancer (SCLC) cell lines but not in five non-small cell lung cancer (NSCLC) cell lines, which are of endodermal origin (Figure 1C).

In summary, *PAX5* expression was detected at the mRNA and protein level in N-type neuroblastoma and SCLC cell lines but not in any of the S-type neuroblastoma, melanoma and non-neural crest-derived NSCLC cell lines, suggesting a role for *PAX5* in the more aggressive phenotype of these cell lines.

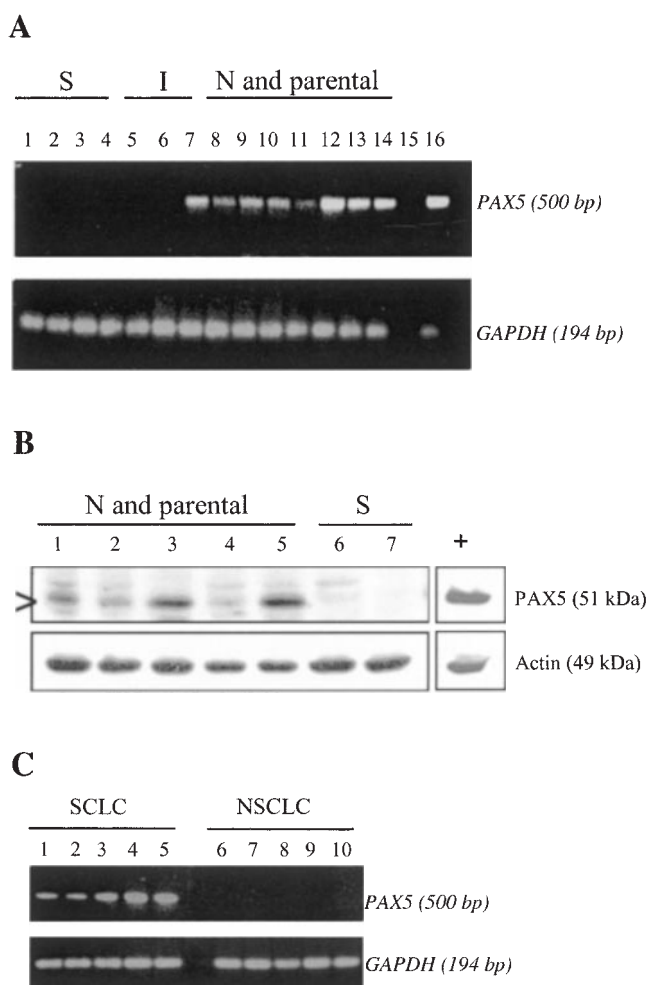


Fig. 1. Expression of *PAX5* in neuroblastoma and lung cancer cell lines. Qualitative analysis of *PAX5* (upper panels) and *GAPDH* (lower panels) expression using RT-PCR with cDNA produced as described. (A) S-type neuroblastoma cell lines SH-EP, CA-2E, SH-310 and WSN (lanes 1–4), I-type cell lines SH-IN, ACN and BE(2)C (lanes 5–7), N-type and parental cell lines LAN-1, SH-SY5Y, Imr32, Kelly, BE(2)M17, LAN-5, SK-N-BE(2) (lanes 8–14) and the B cell lymphoma cell line Kis-1 without (lane 15) and with reverse transcriptase (lane 16) as a control. (B) Immunoblot analysis of *PAX5* expression using 100 µg of total lysate per lane, for the Kis-1 cell line only 60 µg were loaded. The major band of ~50 kDa represents *PAX5* (>), minor bands are non-specific. The actin control is also shown. N-type neuroblastoma cell lines LAN-1, Imr32, LAN-5, SH-SY5Y and SK-N-BE(2) (lanes 1–5) and S-type cell lines SH-EP and CA-2E (lanes 6–7). (C) RT-PCR with cDNA from the SCLC cell lines U1285, NCI-H69, SW2, OH1 and OH3 (lanes 1–5) and the NSCLC cell lines A549, NCI-H125, Calu1, Calu3 and Calu6 (lanes 6–10).

Stable expression of *PAX5* in S-type neuroblastoma cell line CA-2E

To investigate the possibility that the differential expression of *PAX5* in N- but not S-type neuroblastoma cell lines could in part account for their distinctive phenotypes, we generated several independent clones of the S-type neuroblastoma cell line CA-2E with stable expression of *PAX5*. To this end we used the *PAX5*-cDNA expression vector pX-13 and the empty cloning vector pcDNA3.1/Hygro(+) as a control. A number of clones transfected with either vector pX-13 or the control vector were isolated after transfection and limiting dilution. Chromosomal integration of the *PAX5* expression vector or the empty cloning vector was confirmed by vector-specific primers. *PAX5* expression in these clones was determined by immunoblotting (Figure 2A). Three *PAX5* expressing clones (P-16, P-25 and P-28) and three clones transfected with the empty vector (C-43, C-45 and C-50) were randomly selected for further phenotypic analysis. The use of a control vector transfected in parallel and the analysis of three independent clones of each transfected vector should eliminate the possibility of selecting pre-existing clones. To exclude this possibility using an additional method, we performed fluorescence *in situ* hybridization (FISH) experiments with a *MYCN* probe (Vysis, Downers Grove, IL). This experiment showed the presence of two major populations with either three or four *MYCN* copies per cell in the untransfected CA-2E cell line. There was no significant difference in the number of cells with three or four copies between the P and C clones, thereby excluding selection of a subclone with a particular *MYCN* copy number (unpublished data).

PAX5 expression in CA-2E neuroblastoma cells changes cell morphology and increases growth rate

Direct microscopy of cell cultures of *PAX5* expressing CA-2E cells revealed distinct morphologic differences compared with control cells. The latter consisted of flat, epithelial-like cells with a large cytoplasm with no significant difference to untransfected CA-2E cells (Figure 2B). In contrast, *PAX5* expressing cells became smaller, rounder and were less attached to plastic which was closer in morphology to N-type cells (Figure 2C). The observed cell rounding was not due to apoptosis as shown by propidium iodine staining followed by FACS analysis (unpublished data).

Growth kinetics were analyzed by seeding 5000 cells of each of two positive and negative *PAX5* clones and counting cell numbers after different time points. Compared with *PAX5* negative clones, the positive clones grew significantly faster

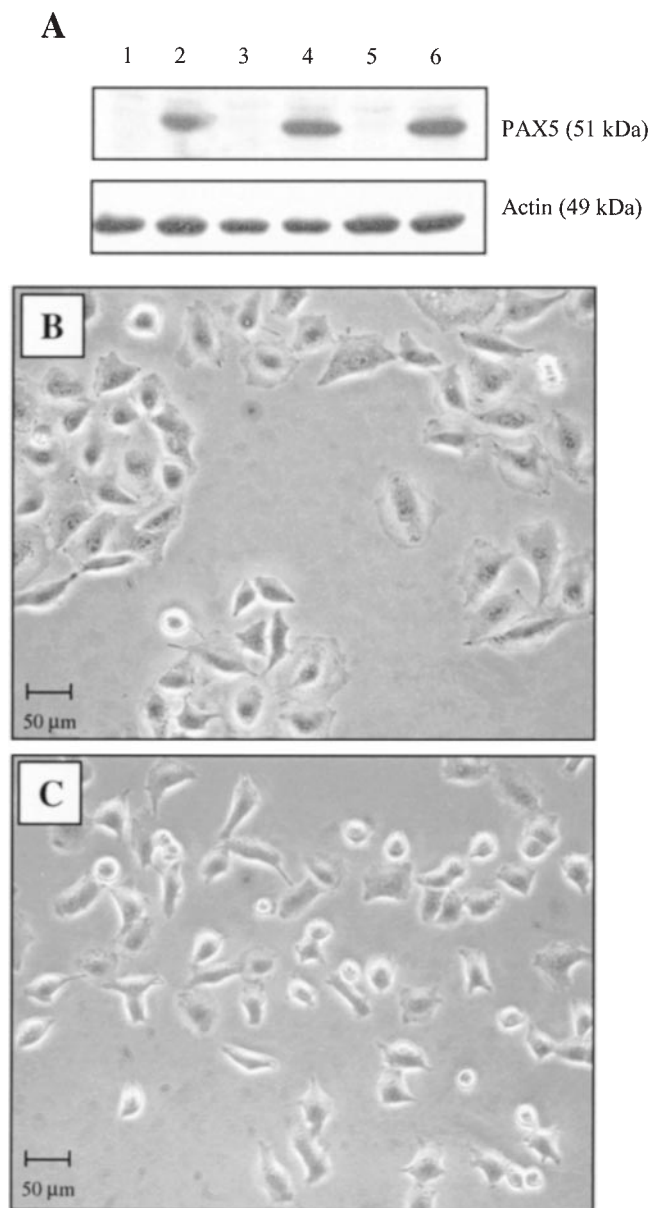


Fig. 2. *PAX5* expression induces morphological changes in CA-2E cells. The S-type CA-2E neuroblastoma cell line was transfected with the human *PAX5* encoding expression vector pX-13 or the empty vector pcDNA3.1/Hygro(+) as a control. Independent clones were isolated by limiting dilution and analyzed for *PAX5* expression and morphology. (A) Immunoblot of 80 µg total cell lysates obtained from the three *PAX5* expressing clones P-16, P-25 and P-28 (lanes 2, 4 and 6) and from the three control clones C-43, C-45 and C-50 (lanes 1, 3 and 5) (upper panel). The equal amount of lysate was verified by a specific staining for actin (lower panel). Photomicrographs of cell cultures from the representative *PAX5* expressing clone P-28 (C) and from the control clone C-45 (B) are also shown and a 50 µm scale is indicated. *PAX5* expressing cells are smaller, rounder and less attached to the culture flask.

and to a higher cell number before the growth curves reached their plateau (Figure 3). Moreover, there was no difference in growth kinetics between the CA-2E cell line and the control vector transfected cell line (unpublished data).

PAX5 expression greatly increases anchorage-independent growth of CA-2E cells

Previous studies have shown that S-type cells isolated from mixed neuroblastoma cell populations are less tumorigenic

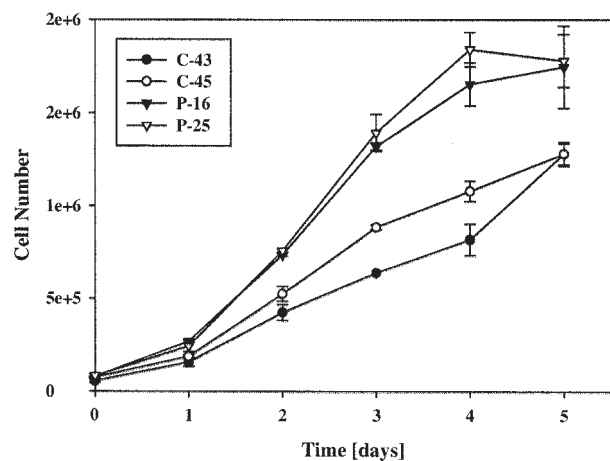


Fig. 3. Increased proliferation of *PAX5* expressing CA-2E cells. 5000 cells of two *PAX5* expressing (P-16 and P-25) and two non-expressing clones (C-43 and C-45) were plated and growth was recorded. Cell numbers were assessed with a Coulter counter at indicated time points. Cells were first counted on day 5, which is set as zero in the figure. The experiment was performed in triplicate for each clone and time point. Means and standard deviations are shown.

than N-type cells, based on their differential ability for colony formation in soft agar assays or tumor formation in nude mice (8). Consistent with these observations CA-2E cells formed nearly no colonies in our soft agar system, in contrast to the N-type cell lines SH-SY5Y and LAN-1 (unpublished data). *PAX5* expressing CA-2E clones formed a large number of colonies that were nevertheless smaller in size compared with colonies formed by the N-type cell lines SH-SY5Y and LAN-1. The control clones were comparable with the wild-type CA-2E cells with almost no colony formation (Figure 4). These observations could be reproduced for all clones in several different experiments. Taken together these results indicate that *PAX5* expression can increase anchorage-independent colony formation when over-expressed in the appropriate cellular background.

PAX5 expression promotes tumor formation in nude mice

In *in vivo* experiments the *PAX5* expressing P-25 and the non-expressing C-43 cells were injected into four CD-1 nude mice each on the lateral flank. Two mice injected with P-25 developed small tumors with a size of 130 and 230 mm³ after 4 months whereas mice injected with C-43 did not form any tumors. To confirm this result in a larger population we injected P-25 cells into seven mice. Although we injected the cells in both flanks only two mice developed tumors with a size of ≤85 mm³. Immunohistochemical analysis of the tumors from both experiments confirmed that they were derived from P-25 as they expressed *PAX5* as well as the CA-2E marker Vimentin (Figure 5A and B). Immunoreactivity for *PAX5* was present in the nucleus and co-localized with Vimentin staining in the same cells.

PAX5 down-regulation with siRNA decreases cell proliferation of N-type neuroblastoma cells

As shown in our growth experiments, stable *PAX5* expression significantly increased the growth rate of CA-2E cells. As N-type cells already express endogenous *PAX5* we exploited the RNA interference (RNAi) technique (31) in order to specifically down-regulate *PAX5* mRNA. We designed an siRNA against *PAX5* mRNA and an scRNA consisting of the same

A

Clone	Colony Number
P-25	91 ± 12
P-16	76 ± 5
P-28	50 ± 7
C-43	6 ± 6
C-45	6 ± 8
C-50	2 ± 2

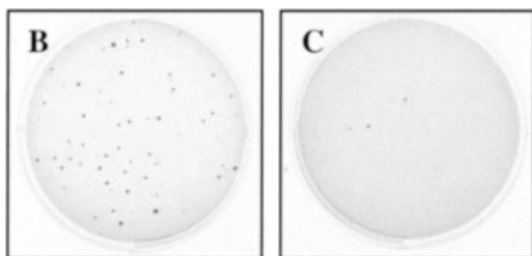


Fig. 4. *PAX5* expressing CA-2E cells are capable of growing anchorage-independently. The soft agar assay was performed with three *PAX5* expressing (P-16, P-25 and P-28) and three *PAX5* non-expressing clones (C-43, C-45 and C-50) derived from the CA-2E neuroblastoma cell line. (A) The data summarized in the table represent the means and standard deviations of a representative experiment carried out in triplicate. The lower panel shows pictures of the *PAX5* expressing clone P-16 (B) and of the non-expressing clone C-43 (C) after staining with 0.01% crystal violet.

bases organized in a randomized combination without any homology to human genomic sequences. The N-type neuroblastoma cell lines LAN-1 and Imr32 as well as the SCLC cell line OH1 were either transfected with siRNA or the same amount of scRNA. The melanoma cell line MML157, which does not express endogenous *PAX5* was used as a control. Immunoblot analysis of cells transfected with siRNA and harvested 72 h after transfection showed a down-regulation of the *PAX5* protein level by 32% in LAN-1 cells and by 12% in Imr32 and OH1 cells when compared with scRNA treatment (Figure 6A–C). Staining for actin was used as a loading control and is also shown.

Proliferation of transfected cells was measured by a MTT assay. A modest but significant decrease in growth of the siRNA transfected cells compared with scRNA transfected cells was first observed 48 h after plating (Figure 6A–C). The effect was greatest in LAN-1 where the *PAX5* down-regulation worked most effective and small in OH1 where *PAC5* was weakly down-regulated. In addition the control cell line MML157 was unaffected by either treatment (Figure 6D).

Discussion

In this study we report the expression of the oncogenic transcription factor *PAX5* in a subset of neuroblastoma cell lines characterized by an aggressive phenotype, the so-called

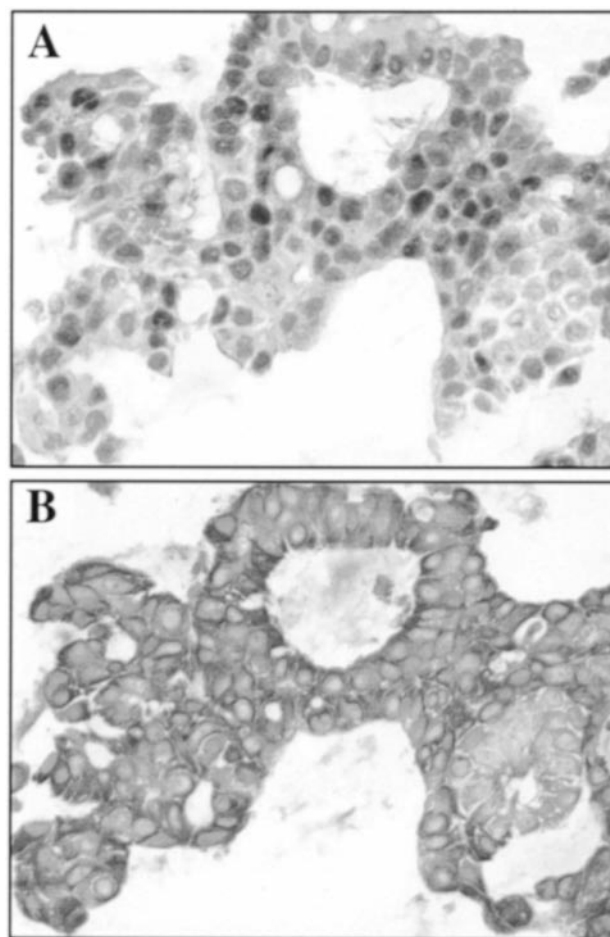


Fig. 5. Tumors established from the CA-2E transfected clone P-25 express markers *PAX5* and Vimentin. Paraffin sections of a xenograft of the *PAX5* expressing CA-2E clone P-25 established in a CD-1 nude mouse are shown. The tumor was isolated 4 months after injection into the lateral flank with a size of 230 mm³. Immunohistochemical stainings for *PAX5* (A) and Vimentin (B) were performed as described. Magnification is 280-fold.

‘N-type’ cells. In contrast, *PAX5* was not expressed in any of the tested S-type neuroblastoma cell lines. Further screening of neural crest-derived tumors demonstrated expression in SCLC but not in melanoma cell lines. The data show a distinct expression pattern of *PAX5* in neural crest-derived tumors with a neuronal or neuroendocrine differentiation, indicating that *PAX5* might be a valuable marker for these derivatives. The lack of expression in both melanoma cell lines and S-type cell lines is consistent with the model of S-type cells being differentiated along the melanocyte pathway (4).

More significantly, the expression of *PAX5* in N-type neuroblastoma cells might be an important determinant of the more malignant phenotype of these cells. To test this hypothesis two approaches were taken. First, *PAX5* was over-expressed in the S-type neuroblastoma cell line CA-2E, resulting in a remarkable phenotypic change. Secondly, *PAX5* was down-regulated in N-type cell lines and SCLC cell line by means of the siRNA technique. A recent report has shown the coexistence of subpopulations with different *MYCN* copy numbers in the neuroblastoma cell line SK-N-SH and the derived cell lines SH-SY5Y (N-type) and SH-EP (S-type). It was suggested that the phenomenon of phenotypic interconversion is likely to result from selection of pre-existing clones that can outgrow

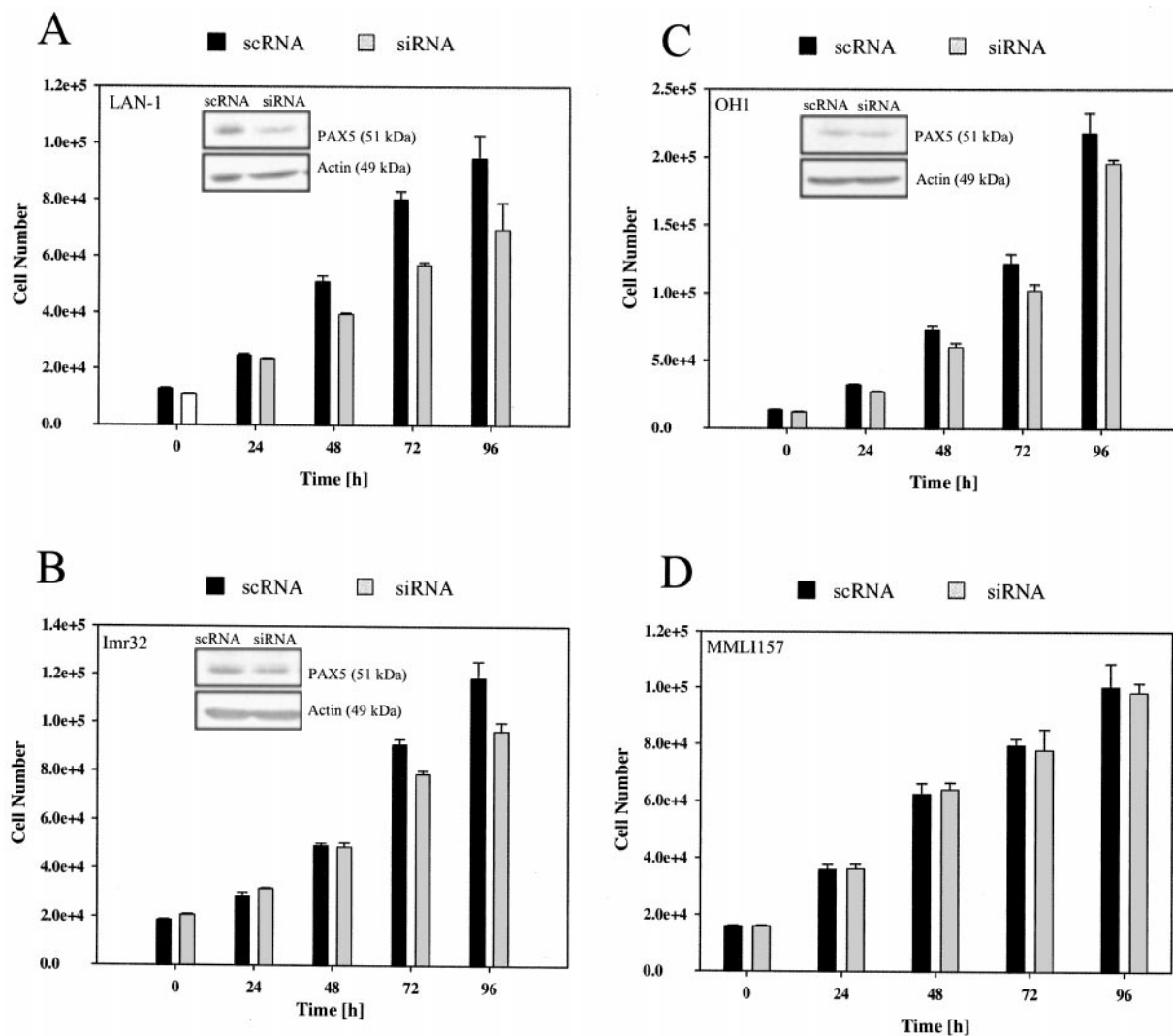


Fig. 6. Down-regulation of *PAX5* expression in N-type neuroblastoma and SCLC cell lines leads to a reduced growth rate. Endogenous *PAX5* expression in the N-type neuroblastoma cell lines LAN-1 (A) and Imr32 (B) as well as in the SCLC cell line OH1 (C) was targeted by transfection of 80 nM of a sequence specific siRNA or 80 nM of a scrambled control scRNA. *PAX5* protein level was determined 72 h after transfection by an immunoblot using 100 μ g from total lysates (A–C). Loading of equal amounts was verified by immunoblotting for actin. The proliferation of transfected cells was assessed using the MTT colorimetric assay at indicated time points (A–C). MML1157 was thereby used as a negative control (D). The number of cells was calculated according to a calibration curve to correlate optical density and cell number. An individual standard curve was constructed for each cell line. The data represent mean values and standard deviations of an experiment carried out in quintuplicate.

under certain conditions (32). The selection of a subclone with a higher *MYCN* copy number could therefore account for the increase in aggressiveness seen in our clones transfected with *PAX5*. To exclude this important possibility we performed FISH analysis with the *MYCN* probe in the untransfected CA-2E cell line and the transfected clones. We could indeed detect the coexistence of two populations with respect to *MYCN* copy number, but no evidence of selection after transfection. Therefore, these results provide evidence that the phenotypic changes observed by us, including the increase of tumorigenicity, are a result of the expression of *PAX5* in CA-2E cells.

The cellular background of the CA-2E cell line proved to be particularly permissive to mediate the effects of *PAX5* expression, most likely because interacting co-factors required for *PAX5* function are present. CA-2E was established from the bone marrow aspirate of a 16-month-old patient with progressive disease (33). The cells have the typical morphology

of S-type cells and do not form colonies in a soft agar assay. *PAX5* expression resulted in a striking increase in colony numbers. The size of the individual colonies, however, was smaller than those formed by the N-type cell line SH-SY5Y. Consistent with this observation, *PAX5* expressing cells formed tumors only with low efficiency in animals. These results are reminiscent of observations made when analysing the transforming capacity of the *PAX3-FKHR* fusion protein (34). Expression of this fusion protein in chicken embryo fibroblasts led to cellular transformation *in vitro*, as shown in focus and soft agar colony assays. In animal experiments, however, no *in vivo* transforming capacity could be demonstrated. It is possible that tumor induction *in vivo* requires additional genetic changes or induction of additional genes (e.g. responsible for neo-angiogenesis) that are not required for colony formation in soft agar. In addition, several other attempts to induce tumors in transgenic models by over-expressing *PAX5* or *PAX3-FKHR* have failed (35–37). These

results could indicate that deregulated expression of *PAX* genes is insufficient to cause tumor formation *in vivo*. However, these results do not exclude an important role for *PAX* gene expression in tumor progression or maintenance. Our observation that targeting of *PAX5* expression in N-type neuroblastoma cell lines decreases proliferation supports this hypothesis.

Our results confirm other observations implicating mouse Pax5 as a positive regulator of cell proliferation (38). In addition down-regulation of *PAX5* in N-type cells affected growth, and we are currently trying to identify the target genes mediating this effect. A novel finding is our observation that *PAX5* expression had a strong effect on S-type cell morphology and adhesion. This suggests that *PAX5* might also regulate expression of cytoskeletal components and/or proteins of extracellular matrix.

Our study is limited by the fact that the effect of *PAX5* over-expression was studied in only one S-type cell line. Nevertheless, the expression data and the effects of *PAX5* expression in the S-type neuroblastoma cell line CA-2E suggest a role for *PAX5* in the molecular pathogenesis of neuroblastoma tumors. Whether *PAX5* might serve as a molecular target for therapy is not known. To investigate this possibility we have started an analysis of *PAX5* expression in tumor samples. Recent findings of widespread expression of *PAX* genes in numerous common tumors and dependence of tumor viability on that expression suggest that *PAX5* and other *PAX* transcription factors might be valuable targets for cancer therapy (39). It is clear however, that the *PAX5* transfected CA-2E cell line is a very useful model system to study the oncogenic function of *PAX5*. For example, the target genes mediating the oncogenic effect of *PAX5* can be identified using microarray technology. Using this technique we have already identified several target genes that are regulated by *PAX5* also in cell lines other than neuroblastoma cells (manuscript in preparation). The results of these studies will provide answers on how *PAX5* performs its oncogenic function.

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